Gastric Invasion by *Trypanosoma cruzi* and Induction of Protective Mucosal Immune Responses

DANIEL F. HOFT,^{1*} PATRICIA L. FARRAR,² KATHERINE KRATZ-OWENS,¹ AND DAWN SHAFFER²

Departments of Internal Medicine¹ and Comparative Medicine,² Saint Louis University Health Sciences Center, St. Louis, Missouri 63110

Received 6 February 1996/Returned for modification 27 March 1996/Accepted 13 May 1996

***Trypanosoma cruzi* is an intracellular parasite transmitted from a reduviid insect vector to humans by exposure of mucosal surfaces to infected insect excreta. We have used an oral challenge murine model that mimics vector-borne transmission to study *T. cruzi* mucosal infection. Although gastric secretions have microbicidal activity against most infectious pathogens, we demonstrate that *T. cruzi* can invade and replicate in the gastric mucosal epithelium. In addition, gastric mucosal invasion appears to be the unique portal of entry for systemic *T. cruzi* infection after oral challenge. The mucosal immune responses stimulated by *T. cruzi* gastric infection are protective against a secondary mucosal parasite challenge. This protective mucosal immunity is associated with increased numbers of lymphocytes that secrete parasite-specific immunoglobulin A. Our results document the first example of systemic microbial invasion through gastric mucosa and suggest the feasibility of a mucosal vaccine designed to prevent infection with this important human pathogen.**

Trypanosoma cruzi is the protozoan parasite that causes Chagas' disease, which affects up to 20 million people in Latin America (53). Most cases of Chagas' disease occur as a result of transmission to humans by a reduviid insect vector (6, 21). The insect deposits infective parasites (IMT) on the surface of the host when it excretes liquid translocated from its foregut to its hindgut by a mechanism designed to concentrate its newly ingested blood meal. Reduviid bugs do not inject IMT into the bloodstream of the host in the way that *Anopheles* mosquitoes transmit malaria, *Phlebotomus* sand flies transmit leishmaniasis, or *Glossina* tsetse flies transmit African trypanosomiasis. *T. cruzi* initiates infection by invading mucosal membranes or other exposed epithelia. After replication within local surface tissues as intracellular amastigote forms, the parasite differentiates into a third life stage, the blood-form trypomastigote, which disseminates throughout the lymphatics and bloodstream, causing high-level parasitemias. During this acute phase of infection, patients generally suffer only minor symptoms and only rarely present with clinical disease. Within weeks to months after the initial infection, immune mechanisms prevent further widespread parasite proliferation, although intracellular parasitism persists for the life of the host. During chronic immune control of the parasite, pathologic lesions occur within cardiac and gastrointestinal tissues of infected individuals. These chronic chagasic lesions are responsible for substantial morbidity and mortality in areas where the disease is endemic.

Because the major forms of Chagas' disease are associated with chronic low-level intracellular infection which persists despite immunity that prevents overwhelming *T. cruzi* replication, a vaccine approach must focus on the complete prevention or eradication of persistent tissue parasitism. Once systemic *T. cruzi* infection occurs, the parasite is known to be capable of suppressing host immune responses (3, 4, 10, 11, 15, 19, 20, 24, 25, 34, 40, 46, 48, 49, 51). Therefore, the prevention of chronic infection may be the only achievable goal of vaccination. The

best method of preventing chronic infection may be to block the initiation of infection. Immune responses induced by vaccination that are capable of inhibiting infection at mucosal surfaces and other exposed epithelia could block the initiation of infection. The relative proportions of vector-borne transmission related to IMT contamination of mucosal surfaces and of subcutaneous infection through open wounds are unknown. Both are likely to be common routes for the initiation of *T. cruzi* infection, and the development of methods to induce local mucosal and subcutaneous immune responses protective against the parasite will be important for vaccine development. Our present work concentrates on investigations of mucosal immunity to *T. cruzi* as one important component of the development of a vaccine directed against Chagas' disease. We have focused on an experimental oral (p.o.) mucosal challenge route because p.o. mucosal infection of both mice and humans has been well documented (1, 5, 8, 13, 14, 23, 31, 43, 44) and is therefore biologically relevant for vaccine studies. Histopathologic studies that document the anatomical route of infection in mice after this p.o. challenge with IMT were completed. In addition, we have evaluated the nature of mucosal immunity at the site of *T. cruzi* invasion after this p.o. IMT challenge.

MATERIALS AND METHODS

Parasites, mice, and challenge protocol. The life cycle of the Tulahuén strain of *T. cruzi* was maintained by passage through mice and the reduviid vector *Dipetalogaster maximus*. To collect IMT, infected reduviid bugs were allowed to feed on anesthetized mice, and then engorged insects were incubated in glass vials for 3 to 4 h as previously described (23). Excreta from multiple reduviids were pooled, and the concentration of IMT was determined by a direct hemacytometer count. IMT preparations were diluted with phosphate-buffered saline (PBS) so that 10 to 40 μ l contained 10,000 to 50,000 parasites. Four- to six-week-old female BALB/cAnNHsd mice were challenged with these IMT preparations by placement of an appropriate volume of parasite suspension in their mouths with a P20 pipetman. Pairs of mice from a group of 12 mice inoculated with 44,400 IMT p.o. were euthanized and necropsied every other day through day 12 postchallenge. Pairs of mice from another group of 10 mice inoculated with 10,000 IMT p.o. were necropsied every 3 days through day 14 postchallenge. Another 18 mice were inoculated with 20,000 to 40,000 IMT p.o. and studied 7 to 28 days postchallenge. For secondary challenge, groups of five mice were inoculated with 20,000 IMT p.o. 6 weeks after a primary p.o. challenge with 20,000 IMT. Murine immune responses do not completely eradicate *T. cruzi* infection after primary oral infection. However, the immune responses induced in immunocompetent BALB/c mice by primary exposure to the parasite do limit

* Corresponding author. Mailing address: Division of Infectious Diseases, Saint Louis University Health Sciences Center, 3635 Vista Ave., FDT-8N, St. Louis, MO 63110. Phone: (314) 577-8648. Fax: (314) 771-3816. Electronic mail address: hoftdf@sluvcu.slu.edu.

systemic *T. cruzi* replication and provide protection against overwhelming progression of the primary infection. In addition, the immune responses present during chronic *T. cruzi* infection have been shown to provide concomitant systemic immunity protective against virulent parenteral secondary challenges with the parasite (2, 7, 18).

Preparation of histopathologic sections. Mice were euthanized by carbon dioxide inhalation at timed intervals within 2 weeks postinoculation. Blood was drawn via cardiocentesis immediately after euthanasia. One drop of blood was placed in a precleaned slide, covered with a coverslip and examined microscopically (20 fields at $\times 400$) for motile *T. cruzi* trypomastigotes. The head, submandibular salivary glands, heart, lungs, gastrointestinal tract (esophagus, stomach, duodenum, jejunum, ileum, cecum, and colon), mesenteric lymph nodes, spleen, liver, kidney, and skeletal muscle (quadriceps) were collected from each animal and fixed in 10% neutral buffered formalin with 1% methyl alcohol (Alban Scientific, Inc., St. Louis, Mo.). Intact mouse heads were decalcified overnight in hydrochloric acid with EDTA (Decalcifier II; Surgipath Medical Industries, Inc., Richmond, Ill.), rinsed with tap water, and returned to 10% neutral buffered formalin-1% methyl alcohol prior to processing and embedding. After decalcification, three sagittal sections were cut from each head (2 mm caudad to upper incisors, adjacent to rostral eye, and adjacent to caudad eye). Continuous longitudinal cross sections were cut from the esophagus, stomach, and duodenum to facilitate microscopic orientation in relation to the margo plicatus, esophageal entrance, and duodenal exit. The residual esophageal, gastric, and duodenal tissues were trimmed longitudinally. The entire small intestine was placed in a Swiss gutroll. Trimmed tissues were processed by gradual dehydration in a graded ethanol solution followed by xylene immersion and were embedded in Paraplast X-tra (Oxford Labware, St. Louis, Mo.). All tissues were fixed in formalin for 2 to 3 days prior to processing and embedding. Serial 5- μ m tissue sections were cut, and adjacent sections were stained with hematoxylin and eosin (H&E) or by the immunohistochemical technique described below for examination by light microscopy.

Immunohistochemical detection of intracellular *T. cruzi* amastigotes. Serial histologic sections of formalin-fixed tissues were stained immunohistochemically to facilitate identification of *T. cruzi* intracellular amastigotes. Sections were heated at 60°C for 45 min to 1 h, deparaffinized in xylene, and rehydrated with a graded series of ethanol-deionized water solutions. Endogenous peroxidase activity was quenched with a 5-min immersion in 3% hydrogen peroxide, and background staining was blocked by a 10-min overlay with 10% fetal bovine serum in PBS. A primary polyclonal antibody (rabbit anti-*T. cruzi* serum) diluted 1:500 in PBS was applied to tissue sections and incubated for 1 h. After a wash with PBS, a 1:1,000 dilution in PBS of goat anti-rabbit antibody reagent conjugated to horseradish peroxidase (Jackson Immunoresearch Laboratories, West Grove, Pa.) was applied for an additional 1-h incubation period. After another wash with PBS, 3-amino-9-ethylcarbazole (Pierce, Rockford, Ill.) was applied for 10 min. All incubations were completed in a warm, moist chamber. After the 3-amino-9-ethylcarbazole substrate was washed away with PBS followed by deionized water, Mayer's hematoxylin (Polyscientific Research and Development Corporation, Bay Shore, N.Y.) was applied for 1 min as a counterstain. Shown in Fig. 1 are liver sections from a normal control mouse and a *T. cruzi*-infected mouse after staining by this immunohistochemical technique. Negative and positive controls were included in each immunohistochemical staining experiment.

GML extraction. Mice were sacrificed by CO₂ narcosis, and whole stomachs were isolated by severing the gastroesophageal junction, the distal pylorus, and the gastric vascular stump. The stomachs were opened, rinsed free of food contents, and minced into 2- to 4-mm squares. In initial experiments, these gastric tissue squares were placed in RPMI medium without additives and shaken vigorously. Mononuclear cells released into the supernatant by this treatment were purified over Percoll density gradients and used as gastric mucosal intraepithelial lymphocytes (IEL). After IEL removal, the residual tissue fragments were digested with 1.5 mg of Dispase II neutral protease (per ml) from *Bacillus polymyxa* (Boehringer Mannheim GmbH, Mannheim, Germany) four times with 15-min incubations at 37°C. Mononuclear cells released into the supernatants were washed free of the Dispase solution, pooled, and purified over Percoll density gradients. These latter preparations were considered representative of the lamina propria lymphocytes. The yields obtained with separate IEL and lamina propria lymphocyte populations were low ($\leq 50,000$ cells for each from 5 to 10 animals). In later experiments, we found that we could increase our yields 10- to 50-fold by digesting whole stomachs with Dispase without the initial IEL purification. Because of these results, we routinely harvested the preparations of total gastric mucosal lymphocytes (GML) for our immunological experiments and for quantification of parasite replication in tissues. Within 1 to 2 weeks after *T. cruzi* p.o. challenge, enlarged lymph nodes attached to the lesser curvature of the stomach were grossly visible. These enlarged nodes were presumed to be the initial lymphoid drainage site from the region of gastric mucosal parasite invasion, and single-cell suspensions (lymph node cells [LNC]) were prepared for study. Single-cell suspensions of spleen cells (SC) were prepared by pushing splenic fragments thru wire mesh and treating with ammonium chloride to lyse erythrocytes.

ELISPOT assay for the detection of *T. cruzi*-specific IgA and IgG responses. Parasite-specific antibody enzyme-linked immunospot (ELISPOT) assays were developed on the basis of the method of Czerkinsky et al. (12). Briefly, culture-derived metacyclic trypomastigotes (CMT) of *T. cruzi* were harvested from par-

asite cultures grown in Grace's medium (45), washed with PBS, and resuspended at a concentration of 10^9 parasites per ml. These CMT suspensions were subjected to three cycles of rapid freezing in liquid N₂ followed by thawing at 37°C. These disrupted suspensions of CMT were filtered through a 0.45- μ m-pore-size filter, and the filtrate was used to coat Millititer HA 96-well microtiter plates lined with nitrocellulose bases (Millipore, Bedford, Mass.). After overnight incubation at 4°C, the wells were washed with sterile PBS and blocked with 10% fetal calf serum, and then fivefold dilutions of mononuclear cells freshly harvested from mucosal and systemic sources were added at a beginning concentration of 10^6 /ml. Mononuclear cells were incubated in these wells coated with *T. cruzi* CMT lysates at 37°C with 5% CO₂ overnight prior to being washed with PBS-0.05% Tween 20. Biotinylated preparations of goat anti-mouse IgA or goat anti-mouse IgG (Jackson Immunoresearch Laboratories) were added to detect parasite-specific antibodies bound to the CMT lysate. The ELISPOT assays were developed with streptavidin conjugated to horseradish peroxidase followed by 3-amino-9-ethylcarbazole substrate precipitation. After drying, the number of spot-forming cells (SFC) was counted with the aid of an Olympus dissecting scope.

ELISPOT assays for the detection of Th1 and Th2 cytokine responses. Cytokine-specific ELISPOT assays were also developed on the basis of the method of Czerkinsky et al. (12). Nitrocellulose-lined Millititer plates were coated with monoclonal antibodies specific for murine gamma interferon (IFN- γ) (R46A2; American Type Culture Collection, Rockville, Md.) and interleukin-4 (IL-4) (11B11; Pharmingen, San Diego, Calif.). PBS containing 10% fetal calf serum was used to block further nonspecific binding. Mononuclear cells previously incubated for 5 days with medium or *T. cruzi* lysate were then harvested, counted, and used to prepare cellular titration curves with fivefold dilutions in cytokine-specific ELISPOT wells. After overnight incubation at 37°C with 5% CO₂, the wells were washed with PBS-0.05% Tween 20, and cytokine-specific antibody reagents were added. IFN- γ ELISPOT assay wells were incubated with a previously described polyclonal rabbit anti-murine recombinant IFN- γ serum (16). A donkey anti-rabbit IgG polyclonal reagent conjugated to horseradish peroxidase (Jackson Immunoresearch Laboratories) was used to detect bound rabbit antibodies. The IL-4 ELISPOT assay mixtures were incubated with a biotinylated anti-murine IL-4 monoclonal antibody (Pharmingen) followed by streptavidin conjugated to horseradish peroxidase. The 3-amino-9-ethylcarbazole substrate was added to visualize SFC. The number of SFC was counted with the aid of an Olympus dissecting scope after the nitrocellulose membranes were allowed to dry.

Quantification of mucosal *T. cruzi* replication. Comparisons of the degrees of parasite replication in different experimental groups were made by examination of immunohistochemically stained mucosal sections from representative animals. However, these examinations allowed qualitative comparisons only. In order to make quantitative comparisons of mucosal parasite replication in different experimental groups, we used a modification of the procedure described by Nunes et al. (36), which has been shown to be correlated with and more sensitive than standard parasitologic methods for the quantification of parasite replication in tissues. Briefly, titrations of GML, LNC, and SC preparations were plated out in 96-well microtiter plates with 200 μ l of liver infusion tryptose broth (Oxoid, Basingstoke, England) containing 0.02 mg of hemin per ml, 100 U of penicillin per ml, 100 μ g of streptomycin per ml, and 10% fetal calf serum (LDNT+ medium) (9, 22) and then incubated at 26°C for 2 months. The initial plating concentration was 5×10^3 mononuclear cells per 200 μ l of LDNT+ medium for GML preparations and 1×10^5 mononuclear cells per 200 μ l of LDNT+ medium for LNC and SC preparations. Threefold dilutions of mononuclear cells were prepared across the 12 wells of a microtiter row in duplicate. The wells were inspected weekly by inverted light microscopy for evidence of *T. cruzi* epimastigote proliferation. The time for development of visible parasites as well as the minimal initial mononuclear cell concentration for each preparation associated with parasite proliferation were recorded. The time for development of visible parasites correlates with initial parasite concentration in a logarithmic fashion related to kinetic growth curves of the organism but in a way that is difficult to quantitate accurately. The minimal initial mononuclear cell concentration associated with parasite proliferation was used to estimate initial parasite concentrations.

RESULTS

Evidence for a progressive gastritis after *T. cruzi* IMT p.o. mucosal challenge. In order to determine the anatomical route of infection after p.o. challenge with *T. cruzi* IMT, we examined H&E-stained tissue sections of mice within the first 2 weeks postchallenge. Initial experiments were conducted with mice challenged p.o. with 1,000 IMT, which is equivalent to 50 times the 50% infective dose for oral IMT challenge (23). We were unable to find evidence of parasitic invasion anywhere within the oropharynx or gastrointestinal tract after this challenge dose. We increased the p.o. IMT challenge dose 10- to 50-fold, and the histopathologic examinations were repeated.

FIG. 1. Immunohistochemical staining with a polyclonal anti-*T. cruzi* antiserum is a sensitive and specific method for the detection of intracellular amastigote parasite forms. (A) Microscopic appearance of normal mouse liver stained by the immunohistochemical technique. (B) Microscopic appearance of an immunohistochemically stained liver section obtained from a mouse infected with *T. cruzi*. Note the magenta color staining individual amastigotes present in panel B only.

FIG. 2. A progressive inflammatory infiltrate develops in the proximal glandular stomach after p.o. IMT challenge. (A) Photomicrograph of a longitudinal section of murine stomach mucosa stained with H&E, prepared from a BALB/c mouse challenged p.o. 4 days previously with *T. cruzi* IMT (magnification, $\times 100$). An early mixed inflammatory infiltrate is evident at the base of the superficial mucosa immediately adjacent to the junction (margo plicatus) between the keratinized stratified squamous epithelium of the more proximal nonglandular stomach and the columnar epithelium lining the true glandular gastric mucosa (arrowhead). The submucosa and muscular layers are normal. (B) Similarly H&E-stained section obtained from a BALB/c mouse 14 days after p.o. challenge with *T. cruzi* IMT (magnification, $\times 100$). The inflammation has progressed to involve the submucosa and underlying muscularis. (C) Higher-power view (magnification, $\times 200$) documenting extension of the inflammation through the muscularis and serosa and into adjacent mesentery.

Again, there was no evidence for inflammation or parasite replication within the mucosa of the oropharynx or esophagus. A mixed inflammatory infiltrate was observed in a region of superficial gastric epithelium by 4 days after p.o. IMT challenge; it was highly localized to the first few centimeters of glandular mucosa distal to the margo plicatus (Fig. 2A). Enlarged lymph nodes adjacent to this region of gastric mucosa were frequently detected within the first week postchallenge. No other significant histopathologic abnormalities were found in any of the other organs examined at these early time points. This pattern of early inflammation occurring in a highly restricted region of the superficial gastric epithelium after oral mucosal challenge was a consistent finding in multiple experiments involving 40 mice. By 14 days after the p.o. IMT challenge, the inflammation within the proximal glandular stomach had become much more severe, including an expanded component of mononuclear cells and extending to involve the gastric muscularis, gastric serosa, and adjacent mesentery (Fig. 2B and C). In addition, motile *T. cruzi* trypomastigotes were routinely observed in direct blood smears by 14 days after the p.o. IMT challenge, and disseminated inflammatory lesions were found in the pancreas, spleen, liver, bone marrow, heart, duodenum, adrenal, brain, and/or skeletal muscle of all animals examined.

Evidence for initial parasite replication within gastric mucosa after *T. cruzi* IMT p.o. challenge. We developed an immunohistochemical staining technique specific for intracellular *T. cruzi* amastigotes to enhance the sensitivity of the detection of parasite proliferation within mucosal tissue. We used this immunostaining technique with tissue sections from the same mice studied by H&E staining. Despite an extensive microscopic review of these immunostained sections, we found no evidence for *T. cruzi* invasion within the oropharynx or esophagus of animals challenged p.o. with any of the doses of IMT used. We did find pseudocysts of amastigotes within sections of gastric mucosa adjacent to the areas of inflammation described above. In Fig. 3, we present results that are representative of the immunostained sections seen in multiple experiments that demonstrate a progression from rare *T. cruzi* pseudocysts present within the superficial gastric mucosa 4 days after p.o. IMT challenge to transmural mucosal parasite replication by day 14 after p.o. IMT challenge. In addition, the enlarged lymph nodes that developed adjacent to regions of gastric *T. cruzi* invasion were found to contain *T. cruzi* pseudocysts as early as 6 days after p.o. IMT challenge (Fig. 4).

Stimulation of parasite-specific IgA and IgG responses in GML after *T. cruzi* IMT p.o. mucosal challenge. In order to determine if gastric invasion by *T. cruzi* IMT stimulated local mucosal antigen-specific B lymphocyte responses, we harvested GML and studied them with antibody ELISPOT assays (Fig. 5). By 2 weeks after p.o. IMT challenge, markedly increased numbers of parasite-specific-IgA-producing cells were detected in GML harvested from infected mice compared with the numbers present in control GML. In addition, increased numbers of parasite-specific-IgG-producing cells were detected in GML, draining LNC, and SC populations harvested from infected mice compared with control GML, LNC, and SC. Similar results were seen with GML, LNC, and SC harvested from mice 4 weeks after p.o. IMT challenge. *T. cruzi*-specific IgA responses were maximal 2 weeks after infection and then decreased by 4 weeks after infection. *T. cruzi*-specific IgG responses continued to increase 4 weeks after infection.

Induction of Th1 and Th2 cytokine responses within gastric mucosa after *T. cruzi* IMT p.o. mucosal challenge. To determine the cytokine profile of antigen-specific T-cell responses stimulated during *T. cruzi* gastric invasion, we performed cy-

tokine-specific ELISPOT assays (Fig. 6). We measured the number of IFN- γ SFC as a marker for the CD4⁺ Th1 lymphocyte profile and the number of IL-4 SFC as a marker for the CD4⁺ Th2 lymphocyte profile. GML, LNC, and SC were harvested from mice 2 weeks after p.o. challenge with IMT and from age- and sex-matched control mice, incubated in vitro with medium or *T. cruzi* lysate for 5 days to stimulate secondary immune responses, and added to cytokine-specific ELISPOT assay mixtures. The draining gastric LNC harvested from infected mice contained increased numbers of *T. cruzi*-specific-IFN- γ -producing cells compared with control LNC. These increased IFN- γ responses in infected LNC appeared to be antigen-specific memory immune responses, because they were detected after secondary in vitro stimulation with parasite lysate but not after incubation with medium alone. In contrast to these results, GML and SC from these infected mice appeared to spontaneously secrete IFN- γ without the need for in vitro secondary parasite lysate stimulation. Live *T. cruzi* trypomastigotes were frequently detected by the end of the 5-day culture period in GML and SC preparations harvested from infected mice, and therefore it is difficult to rule out the possibility that these IFN- γ -secreting cells were responding in an antigen-specific manner to parasite antigens expressed by infected cells.

We measured the number of IL-4-secreting lymphocytes in the same mononuclear cell preparations after in vitro stimulation either with medium alone or with exogenously added parasite lysate. We have not detected increased numbers of IL-4-producing cells in GML harvested from *T. cruzi*-challenged mice compared with control mice. However, both the draining gastric LNC and SC of *T. cruzi*-infected mice were found to contain increased numbers of antigen-specific-IL-4-secreting cells compared with negative control cell populations.

Concomitant immunity induced in gastric mucosa after *T. cruzi* IMT p.o. mucosal challenge. We next investigated the possibility that mucosal immune responses stimulated by gastric mucosal invasion with *T. cruzi* IMT were protective against a secondary p.o. IMT challenge. Mice were challenged p.o. with 20,000 IMT and then again 6 weeks later with a second p.o. dose of 20,000 IMT. Age- and sex-matched naive controls were administered a primary p.o. IMT challenge with the same IMT preparations used for secondary challenge. The *T. cruzi*-specific immunohistochemical staining technique described above was used to evaluate the levels of gastric mucosal parasite replication in representative mice from groups that had received primary versus secondary p.o. IMT challenge 2 weeks earlier. We could detect intracellular amastigotes within the gastric mucosa of mice in the primary challenge groups but not in the gastric mucosa of mice in the secondary challenge groups (data not shown). In addition, the tissue sections obtained from mice in the secondary challenge groups demonstrated a much higher number of inflammatory cells throughout the gastric mucosa. These results suggested that the natural mucosal immunity stimulated by gastric infection 6 weeks earlier resulted in a more rapid and massive recruitment of inflammatory cells into gastric mucosa that was protective against a secondary mucosal challenge. To quantitate the levels of parasite replication in the gastric mucosa, lymph nodes, and spleen after primary and secondary oral IMT challenge, titrations of GML, LNC, and SC suspensions were cultured in standard axenic parasite medium (LDNT+ medium) and monitored for the development of proliferating parasites. These quantitative cultures provided conclusive evidence for the presence of secondary mucosal immune responses protective against *T. cruzi* mucosal invasion (Table 1). We were unable to

FIG. 3. *T. cruzi* gastric invasion is confirmed by the detection of intracellular parasite replication in the regions of progressive inflammation seen by H&E staining. (A) High-power view (magnification, $\times 400$) of a gastric mucosal section stained by the immunohistochemical method (see Fig. 1). Within the superficial gastric mucosal epithelium are pseudocysts of intracellular *T. cruzi* amastigotes. (B) Lower-power view (magnification, $\times 200$) of an immunostained section adjacent to the section shown in Fig. 2B from a mouse 14 days after p.o. challenge with *T. cruzi* IMT.

detect parasites in wells seeded with GML and LNC harvested from mice after the secondary oral IMT challenge. However, we were able to grow parasites from the GML and LNC harvested from mice after primary oral IMT challenge. The titration cultures indicated that GML and LNC harvested from mice after primary challenge contained at least 18- to 83-fold more parasites than GML and LNC harvested after secondary challenge. These results demonstrated that the immune responses stimulated by primary *T. cruzi* gastric infection provide concomitant immunity protective against a secondary gastric challenge with the parasite.

Increased parasite-specific gastric mucosal IgA responses associated with concomitant immunity. To identify immune responses associated with the protective concomitant immunity stimulated by gastric mucosal *T. cruzi* infection, we compared the levels of parasite-specific IgA and cytokine responses in lymphocytes harvested from mice after secondary p.o. IMT challenge with the levels of these responses detected after primary p.o. IMT challenge. We found that the only remarkable difference between lymphocytes harvested from mice after secondary challenge and lymphocytes harvested after primary challenge was in the numbers of parasite-specific-IgA-producing cells present within the gastric mucosa. The GML harvested from mice after secondary p.o. challenge with IMT contained approximately fourfold more parasite-specific-IgA-producing cells than the GML harvested from mice after primary p.o. IMT challenge (Fig. 7). The numbers of parasite-specific-IgG- and -cytokine-secreting cells were similar in all lymphocyte preparations harvested from mice after primary and secondary challenges (data not shown).

DISCUSSION

In our initial pathologic investigations, we examined sections of mice that had been challenged p.o. with 1,000 IMT, a dose previously found to be minimally sufficient for 100% infectivity of BALB/c mice (23). We were unable to find evidence of inflammation or parasite replication anywhere along the gastrointestinal tract after this relatively low-level challenge protocol. It is possible that the numbers of infected cells and the level of associated inflammation were missed in the random intermittent sections prepared for analysis. It is also possible that *T. cruzi* IMT can actively inhibit the secretion of inflammatory mediators such as IL-8 under conditions in which limited epithelial cell damage occurs as a result of only small numbers of developing pseudocysts. *T. cruzi* is well known to have immunosuppressive effects on the host (3, 4, 10, 11, 15, 17, 19, 20, 24, 25, 34, 40, 46, 48, 49, 51), which may include anti-inflammatory effects. Future studies will need to address this issue.

After increasing the p.o. challenge dose 10- to 50-fold, we were able to find evidence for obvious invasion and inflammation within the proximal glandular stomach. These p.o. challenges with higher doses of IMT consisted of no more than 30 to 40 μ l of diluted or undiluted reduviid excreta, volumes that could easily be ingested by susceptible animals and humans during natural vector-borne transmission of *T. cruzi*. Within 4 days after p.o. IMT challenge, recruitment of a mixed inflammatory-cell response, including both polymorphonuclear and mononuclear cells, was detected. By the end of 2 weeks, the

composition of the inflammatory infiltrate had changed to consist predominantly of mononuclear cells. At this 2-week time point after p.o. IMT challenge, *T. cruzi*-specific B and T lymphocytes were detected in gastric mucosal tissue. It is unclear whether these parasite-specific lymphocytes represent the recruitment of peripherally circulating cells or the expansion of lymphocytes resident within the gastric mucosa. Most likely, both of these possibilities are partially correct.

During primary challenge with IMT p.o., the inflammatory response was unsuccessful in containing the parasite infection to the gastric mucosa, leading to systemic infection. In fact, the tissue destruction related to the inflammatory response may aid in dissemination of the parasite infection by causing a disruption of the serosal surface of the stomach, allowing trypomastigotes released from infected cells to enter adjacent structures within the peritoneal cavity. However, a more rapid inflammatory response recruited after secondary p.o. IMT challenge was associated with increased parasite-specific immunity, and it was able to limit parasite replication and may have prevented the secondary systemic spread of *T. cruzi*. It will be important to determine if mucosal vaccination protocols capable of inducing the same subsets of immune responses present during concomitant immunity, perhaps through the use of live vectors or parasite antigens mixed with adjuvants, can promote the same level of protection against a primary p.o. IMT challenge.

The more sensitive immunohistochemical analysis technique specific for intracellular *T. cruzi* amastigotes made it possible to scan large regions of tissue sections on low microscopic power ($\times 200$) relatively rapidly for evidence of parasite infection. The immunohistochemical detection of parasite replication in the proximal glandular stomach mucosa, and not in other areas throughout the gastrointestinal tract, strongly confirms the conclusion drawn from the H&E-stained sections that *T. cruzi* initiates systemic infection after the p.o. IMT challenge protocol by a highly focal gastric mucosal invasion. *Helicobacter pylori* has recently gained attention as an important gastric pathogen (reviewed in references 35 and 37), but this organism is not an intracellular pathogen and does not systemically spread by invading the gastric mucosa. Our results constitute the first evidence for gastric invasion by a nonviral intracellular pathogen.

Gastric infection with *T. cruzi* was found to stimulate mucosal B lymphocyte responses, including both parasite-specific IgA and IgG production. These parasite-specific antibody responses were largely restricted to the gastric mucosal lymphocytes, with 50- to 100-fold-higher numbers of IgA- and IgG-secreting cells present in GML harvested from infected mice compared with the numbers present in GML harvested from control mice. These results indicate that during the first month after *T. cruzi* gastric invasion, parasite-specific B-lymphocyte responses are predominantly induced in or recruited to mucosal tissue and not the draining lymph nodes or spleen. This mucosal concentration of antigen-specific B lymphocytes may reflect a higher load of parasite replication within mucosal tissue, resulting in increased local antigen stimulation. However, it is believed that the inductive sites for mucosal immune responses in the gastrointestinal tract are limited to the Peyer's patches in the small bowel (29). Inductive sites in gastric mu-

FIG. 4. *T. cruzi* spreads rapidly from gastric mucosa to the local draining lymph nodes. (A) Section of gastric mucosa after immunostaining, with an enlarged lymph node adherent to the gastric serosal surface along the distal aspect of the lesser curvature (magnification, $\times 50$). (B) Higher-power view (magnification, $\times 400$) of the same lymph node shown in panel A, demonstrating a *T. cruzi* pseudocyst. This section was obtained from a mouse 6 days after p.o. IMT challenge. The pseudocyst of intracellular amastigotes detected probably represents one of the first secondary cycles of parasite replication after gastric invasion.

FIG. 5. *T. cruzi* gastric invasion is associated with the induction of parasite-specific-antibody-producing cells in gastric mucosa. A freeze-thawed parasite lysate was used to coat ELISPOT assay plates as described in Materials and Methods, and mononuclear cells freshly harvested from the gastric mucosa, draining lymph nodes, and spleen were added for overnight incubation at 37°C with 5% CO₂. After removal of cells, the wells were developed with reagents resulting in precipitated spots corresponding to regions of parasite-specific antibody production by individual lymphocytes (SFC). (A) IgA-producing SFC; (B) IgG-producing SFC. IGML, GML harvested from infected mice; NGML, GML harvested from control mice; ILNC, LNC from infected mice; NLNC, LNC from control mice; ISC, SC from infected mice; NSC, SC from control mice; PI, postinfection. The results (means $\pm$ standard deviations) were derived from one set of concurrent assays involving five mice per group that were representative of three separate experiments.

A

B

FIG. 6. Induction of T-cell cytokine responses after *T. cruzi* gastric invasion. Mononuclear cells were harvested from the gastric mucosa, draining lymph nodes, and spleen; stimulated for 5 days in vitro with medium alone or parasite lysate; and then used in ELISPOT assays specific for IFN- γ (A) and IL-4 (B). IGML, GML harvested from infected mice; NGML, GML harvested from control mice; ILNC, LNC from infected mice; NLNC, LNC from control mice; ISC, SC from infected mice; NSC, SC from control mice. The results (means $\pm$ standard deviations) were derived from one set of concurrent assays involving five mice per group that were representative of three separate experiments and were obtained 14 days after p.o. IMT challenge.

TABLE 1. Quantification of *T. cruzi* replication in tissues after primary and secondary p.o. IMT challenges^a

Source of mononuclear cells	Minimal cell concn (no./ml) with parasites ^b	Minimal estimate of parasites/million mononuclear cells ^c
Gastric mucosa after primary challenge	60	≥16,667
Gastric mucosa after secondary challenge	>5,000 ^d	<200
Draining lymph nodes after primary challenge	7,350	≥180
Draining lymph nodes after secondary challenge	>100,000 ^d	<10
Spleen after primary challenge	1,200	≥833
Spleen after secondary challenge	100,000	≥10

^a Threefold dilutions in duplicate of mononuclear cell preparations harvested from mice 2 weeks after p.o. challenge with 20,000 IMT were prepared in LDNT+ medium and incubated for 2 months at 26°C. Wells were inspected by inverted microscopy weekly for evidence of proliferating parasites.

^b The minimal concentration of mononuclear cells for each preparation associated with parasite proliferation is shown. Duplicates were consistent within one dilution.

^c Minimal estimate of parasites/million cells = 1 million ÷ minimal cell concentration with parasites.

^d The maximum concentration of cells plated was not associated with parasite proliferation.

cosa have not been described. Cytokine production associated with the marked transmural inflammatory response that develops in gastric tissue may aid in the recruitment of peripheral B lymphocytes primed in the classical inductive lymphoid tissue in Peyer's patches. We were unable to find evidence for *T. cruzi* replication or germinal center formation in the Peyer's patches of mice, nor could we detect parasite-specific antibody responses in ELISPOT assays with lymphocytes harvested from Peyer's patches after p.o. IMT challenge (data not shown). However, it is possible that antigens released by dead parasites reach the small bowel, are transported across M cells into Peyer's patches, and prime a limited number of B cells, which circulate and expand when recruited to infected gastric tissue. This is an important issue with regard to vaccine development that we are currently trying to address.

Recent evidence has shown that two populations of CD4⁺

T-helper (Th) lymphocytes that produce distinct profiles of cytokines after antigenic stimulation can be differentially stimulated and can have opposing effects on resistance and susceptibility (26, 32, 33, 41, 42, 55). Th1 cells, which produce IFN-γ after antigenic stimulation, are increased in animals resistant to infection with several different intracellular parasites. Th2 cells secrete IL-4 after antigenic stimulation and are preferentially expanded in mice with increased susceptibility to intracellular parasite infection. However, these studies focused on parasite infections that are not transmitted mucosally. It is generally believed that CD4⁺ Th2 lymphocyte responses predominate over CD4⁺ Th1 responses in mucosal immune responses (30). IL-4 and IL-6 produced by Th2 cells are thought to be important for the induction of antigen-specific-IgA-secreting mucosal B lymphocytes. We detected IFN-γ-producing cells, but not IL-4-producing cells, in the GML harvested from

FIG. 7. The protective concomitant immunity effective against secondary p.o. IMT challenge is associated with increased *T. cruzi*-specific IgA responses. The levels of parasite-specific IgA SFC in BALB/c mice after primary challenge were compared with those after secondary challenge by use of the ELISPOT assay described in the legend to Fig. 5. The secondary challenge was given 6 weeks prior to the secondary challenge. IX2GML, GML harvested 2 weeks after secondary challenge; IX1GML, GML harvested 2 weeks after primary challenge; IX2LNC, LNC harvested 2 weeks after secondary challenge; IX1LNC, LNC harvested 2 weeks after primary challenge; IX2SC, SC harvested 2 weeks after secondary challenge; IX1SC, SC harvested 2 weeks after primary challenge. The results (means ± standard deviations) were derived from one set of concurrent assays involving five mice per group that were representative of three separate experiments.

infected mice. Future studies are needed to determine whether these IFN- γ responses represent a predominance of CD4⁺ Th1 cells in the mucosal immune response to *T. cruzi*.

IFN- γ has been identified as a resistance factor in *T. cruzi* infections (38, 54). The administration of recombinant IFN- γ to mice increases their resistance (27, 39), while the neutralization of IFN- γ in vivo with monoclonal antibodies increases susceptibility (28, 52). We have recently demonstrated that antigen-specific IFN- γ responses are increased during infection in resistant B6 mice compared with susceptible BALB/c mice, and these increased responses occur with kinetics that imply a causative role in resistance (17). These results suggest that CD4⁺ Th1 lymphocytes are involved in systemic resistance to *T. cruzi*, because the experimental conditions used for induction of IFN- γ would be unlikely to stimulate either CD8⁺ cytotoxic T lymphocytes or NK cells, two other cell types known to produce IFN- γ . In our present work, the source for the mucosal IFN- γ responses is unclear. The fact that IFN- γ SFC were detected in mucosal lymphocyte preparations incubated with medium alone suggests that NK cells or some other nonspecific immune cell type could have been the source for these responses. However, parasite-infected cells were seen in cultures of GML by the end of the 5-day incubation period and may have provided ample *T. cruzi* antigen-specific stimulation in vitro despite the absence of exogenous parasite lysate. The release of antigens from dead or viable extracellular parasites could have provided suitable molecules for the exogenous pathway of antigen presentation to CD4⁺ T lymphocytes. Alternatively, intracellular replicating parasites could have provided antigens to the endogenous pathway of antigen presentation for the stimulation of CD8⁺ T lymphocytes.

The lack of detectable IL-4 responses in mucosal lymphocytes is unlikely to be related to a problem of sensitivity of the IL-4 ELISPOT assay, as this assay is capable of detecting a single IL-4-producing cell, and IL-4 responses were detected in draining lymph nodes and spleen with this assay. Despite the lack of mucosal IL-4 responses, we did find high levels of parasite-specific-IgA-producing B lymphocytes within mucosal tissue. Perhaps the IL-4 responses detected in the draining gastric lymph nodes represent Th2 cells primed by infection that were capable of providing the B-cell help necessary for mucosal IgA production.

Our studies of concomitant mucosal immunity suggest that mucosal IgA responses can be protective against *T. cruzi* infection. We do not believe that the induction of mucosal immune responses alone by vaccination will be sufficient to protect humans from all forms of vector-borne and parenteral transmission of *T. cruzi* infection. After all, IMT can invade subcutaneously through open wounds, and mammalian forms of the parasite can be transmitted by transfusion or transplantation. Our work and that of other investigators cited above indicate that CD4⁺ Th1 responses are important for protective immunity. The work of Tarleton et al. (50), with β -2 microglobulin-deficient mice, strongly suggests that CD8⁺ T lymphocytes also contribute to *T. cruzi* resistance. Therefore, we believe that an effective vaccine protocol capable of preventing chronic infection with *T. cruzi* infection must target multiple subsets of immunity. Our present data indicate that the induction of gastric mucosal immunity will be an important component of a vaccine to prevent Chagas' disease.

Finally, our results may be relevant to the pathogenic mechanism of megaesophagus, an important form of chronic Chagas' disease. It is believed that the development of chagasic megaesophagus is associated with the level of damage to the muscular layers harboring the myenteric plexi that innervate the esophagus (47). In regions where the disease is endemic, it

is likely that repeated exposure to IMT excreted by reduviid vectors is common. Repeated ingestion of IMT with subsequent invasion at or near the gastroesophageal junction may promote chronic inflammatory responses or recurrent boosts of concomitant immunity that result in destruction of the myenteric plexi and the initiation of megadisease.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grant AI-34912.

We thank L. V. Kirchhoff for his gift of reduviid bugs and for his many useful discussions over the years. We also thank J. R. McGhee and H. Kiyono, and their colleagues in the Mucosal Immune Research Group at the University of Alabama, for their help in the initiation of our mucosal immune studies. Finally, we thank Sharon Walsh and Susan Hay for assistance with the preparation of tissue sections.

REFERENCES

- Amato Neto, V., L. Matsubara, R. Campos, A. A. Moreira, P. L. Pinto, R. Faccioli, and M. Zugaib. 1992. *Trypanosoma cruzi* in the milk of women with chronic Chagas disease. *Rev. Hosp. Clin. Fac. Med. Sao Paulo* 47:10-11.
- Araujo, F. 1989. Development of resistance to *Trypanosoma cruzi* in mice depends on a viable population of L3T4⁺ (CD4⁺) T lymphocytes. *Infect. Immun.* 57:2246-2248.
- Beltz, L. A., F. Kierszenbaum, and M. B. Szein. 1989. Selective suppressive effects of *Trypanosoma cruzi* on activated human lymphocytes. *Infect. Immun.* 57:2301-2305.
- Beltz, L. A., M. B. Szein, and F. Kierszenbaum. 1988. Novel mechanism for *Trypanosoma cruzi*-induced suppression of human lymphocytes: Inhibition of IL-2 receptor expression. *J. Immunol.* 141:289-294.
- Bittencourt, A. L., M. Sadigursky, A. A. Silva, C. A. S. Menezes, M. M. M. Marianetti, S. C. Guerra, and I. Sherlock. 1988. Evaluation of Chagas' disease transmission through breast-feeding. *Mem. Inst. Oswaldo Cruz* 83:37-39.
- Brener, Z. 1973. Biology of *Trypanosoma cruzi*. *Annu. Rev. Microbiol.* 27:347-382.
- Brener, Z. 1980. Immunity to *Trypanosoma cruzi*. *Adv. Parasitol.* 18:247-319.
- Calvo Mendez, M. L., B. Nogueira Torres, and R. Alejandro Aguilar. 1992. La via oral: una puerta de acceso para *Trypanosoma cruzi*. *Rev. Lat-amer. Microbiol.* 34:39-42.
- Camargo, E. P. 1964. Growth and differentiation in *Trypanosoma cruzi*. I. Origin of metacyclic trypanosomes in liquid media. *Rev. Inst. Med. Trop. Sao Paulo* 6:93-100.
- Choromanski, L., and R. E. Kuhn. 1989. Time-dependent mode of immunization augments suppressed antibody responses in spleen cell cultures from mice experimentally infected with *Trypanosoma cruzi*. *Int. J. Parasitol.* 19:935-938.
- Choromanski, L., and R. E. Kuhn. 1990. Augmentation of suppressed antibody responses in mice during experimental Chagas' disease by T helper cells activated in a time-dependent mode of immunization. *J. Protozool.* 37:388-392.
- Czerkinsky, C. C., L.-A. Nilsson, H. Nygren, O. Ouchterlony, and A. Tarkowski. 1983. A solid phase enzyme-linked immunospot (ELISPOT) assay for enumeration of antibody secreting cells. *J. Immunol. Methods* 65:109-122.
- Dias, J. C. P. 1979. Mecanismos de transmissao, p. 152-174. In Z. Brener and Z. Andrade (ed.), *Trypanosoma cruzi e Doenca de Chagas*. Guanabara Koogan, Rio de Janeiro, Brazil.
- Gus, I., M. E. Molon, and A. P. Bueno. 1993. Chagas disease—review of 8 simultaneous cases of acute Chagas myocarditis: 25 years later. *Arq. Bras. Cardiol.* 60:99-101.
- Hayes, M. M., and F. Kierszenbaum. 1981. Experimental Chagas' disease: kinetics of lymphocyte responses and immunological control of the transition from acute to chronic *Trypanosoma cruzi* infection. *Infect Immun* 31:1117-1124.
- Hoft, D. F., R. G. Lynch, and L. V. Kirchhoff. 1993. Kinetic analysis of antigen-specific immune responses in resistant and susceptible mice during infection with *Trypanosoma cruzi*. *J. Immunol.* 151:7038-7047.
- Hoft, D. F., R. G. Lynch, and L. V. Kirchhoff. 1993. Unpublished data.
- Kierszenbaum, F. 1989. Chagas' disease, p. 173-195. In F. Y. Liew (ed.), *Vaccination strategies of tropical diseases*. CRC Press, Boca Raton, Fla.
- Kierszenbaum, F., W. R. Cuna, L. A. Beltz, and M. B. Szein. 1989. *Trypanosoma cruzi* reduces the number of high-affinity IL-2 receptors on activated human lymphocytes by suppressing the expression of the p55 and p70 receptor components. *J. Immunol.* 143:275-279.
- Kierszenbaum, F., W. R. Cuna, L. A. Beltz, and M. B. Szein. 1990. Trypanosomal immunosuppressive factor: a secretion product(s) of *Trypanosoma cruzi* that inhibits proliferation and IL-2 receptor expression by activated

- human peripheral blood mononuclear cells. *J. Immunol.* **144**:4000–4004.
21. Kirchhoff, L. V. 1995. *Trypanosoma* species (American trypanosomiasis, Chagas disease): biology of trypanosomes, p. 2442–2450. In G. L. Mandell, J. E. Bennett, and R. Dolin (ed.), *Principles and practice of infectious diseases*. Churchill Livingstone, New York.
 22. Kirchhoff, L. V., S. Hieny, M. Shiver, D. Snary, and A. Sher. 1984. Cryptic epitope explains the failure of a monoclonal antibody to bind to certain isolates of *Trypanosoma cruzi*. *J. Immunol.* **133**:2731–2735.
 23. Kirchhoff, L. V., and D. F. Hoft. 1990. Immunization and challenge of mice with insect-derived metacyclic trypomastigotes of *Trypanosoma cruzi*. *Parasite Immunol.* **12**:65–74.
 24. Liew, F. Y., J. A. Schmidt, D. S. Liu, S. M. Millott, M. T. Scott, J. S. Dhaliwal, and S. L. Croft. 1988. Suppressive substance produced by T cells from mice chronically infected with *Trypanosoma cruzi*. *J. Immunol.* **140**:969–973.
 25. Liew, F. Y., M. T. Scott, D. S. Liu, and S. L. Croft. 1987. Suppressive substance produced by T cells from mice chronically infected with *Trypanosoma cruzi*. *J. Immunol.* **139**:2452–2457.
 26. Maggi, E., P. Biswas, G. F. Del Prete, P. Parronchi, D. Macchia, C. Simonelli, L. Emmi, M. De Carli, A. Tiri, M. Ricci, and S. Romagnani. 1991. Accumulation of Th-2-like helper T cells in the conjunctiva of patients with vernal conjunctivitis. *J. Immunol.* **146**:1169–1174.
 27. McCabe, R., S. Meagher, and B. Mullins. 1991. Gamma interferon suppresses acute and chronic *Trypanosoma cruzi* infection in cyclosporin-treated mice. *Infect. Immun.* **59**:1633–1638.
 28. McCabe, R. E., S. G. Meagher, and B. T. Mullins. 1991. Endogenous interferon-gamma, macrophage activation, and murine host defense against acute infection with *Trypanosoma cruzi*. *J. Infect. Dis.* **163**:912–915.
 29. McGhee, J. R., J. Mestecky, M. T. Dertzbaugh, J. H. Eldridge, M. Hirasawa, and H. Kiyono. 1992. The mucosal immune system: from fundamental concepts to vaccine development. *Vaccine* **10**:75–88.
 30. McGhee, J. R., J. Mestecky, C. O. Elson, and H. Kiyono. 1989. Regulation of IgA synthesis and immune response by T cells and interleukins. *J. Clin. Immunol.* **9**:175–199.
 31. Miles, M. A. 1972. *Trypanosoma cruzi*-milk transmission of infection and immunity from mother to young. *Parasitology* **65**:1–9.
 32. Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* **136**:2348–2357.
 33. Mosmann, T. R., J. H. Schumacher, N. F. Street, R. Budd, A. O'Garra, T. A. T. Fong, M. W. Bond, K. W. M. Moore, A. Sher, and D. F. Fiorentino. 1991. Diversity of cytokine synthesis and function of mouse CD4+ T cells. *Immunol. Rev.* **123**:209–229.
 34. Nabors, G. S., and R. L. Tarleton. 1991. Differential control of IFN-gamma and IL-2 production during *Trypanosoma cruzi* infection. *J. Immunol.* **146**:3591–3598.
 35. National Institutes of Health. 1995. NIH Consensus Conference. *Helicobacter pylori* in peptic ulcer disease. NIH Consensus Development Panel on *Helicobacter pylori* in peptic ulcer disease. *JAMA* **273**:376.
 36. Nunes, M. P., S. G. Coutinho, J. A. Louis, and W. J. S. Souza. 1990. *Trypanosoma cruzi*: quantification tissues of experimentally infected mice by limiting dilution analysis. *Exp. Parasitol.* **70**:186–192.
 37. Parsonnet, J., S. Hansen, L. Rodriguez, A. B. Gelb, R. A. Warnke, E. Jellum, N. Orentreich, J. H. Vogelstein, and G. D. Friedman. 1994. *Helicobacter pylori* infection and gastric lymphoma. *N. Engl. J. Med.* **330**:1310–1311.
 38. Plata, F., F. Wietzerbin, F. G. Pons, E. Falcoff, and H. Eisen. 1984. Synergistic protection by specific antibodies and interferon against infection by *Trypanosoma cruzi* in vitro. *Eur. J. Immunol.* **14**:930.
 39. Reed, S. 1988. In vivo administration of recombinant IFN-gamma induces macrophage activation, and prevents acute disease, immune suppression, and death in experimental *Trypanosoma cruzi* infections. *J. Immunol.* **140**:4342–4347.
 40. Rowland, E. C., and R. E. Kuhn. 1978. Suppression of cellular responses in mice during *Trypanosoma cruzi* infections. *Infect. Immun.* **20**:393–397.
 41. Salgame, P., J. S. Abrams, C. Clayberger, H. Goldstein, J. Convit, R. L. Modlin, and B. R. Bloom. 1991. Differing lymphokine profiles of functional subsets of human CD4 and CD8 T cell clones. *Science* **254**:279–282.
 42. Scott, P., E. Pearce, A. W. Cheever, R. L. Coffman, and A. Sher. 1989. Role of cytokines and CD4+ T-cell subsets in the regulation of parasite immunity and disease. *Immunol. Rev.* **112**:161–182.
 43. Shaw, J., R. Lainson, and H. Faria. 1969. Consideracoes sobre a epidemiologia dos primeiros casos autocones de doenca de Chagas registrados em Belem, Para, Brasil. *Rev. S. Publ. (Sao Paulo)* **3**:153–157.
 44. Shikanai-Yasuda, M. A., C. B. Marcondes, L. A. Guedes, G. S. Siqueira, A. A. Barone, J. C. P. Dias, V. Amato Neto, J. E. Tolezano, B. A. Peres, E. R. Arruda, Jr., M. H. Lopes, M. Shiroma, and E. Chapadeiro. 1991. Possible oral transmission of acute Chagas disease in Brazil. *Rev. Inst. Med. Trop. Sao Paulo* **33**:351–357.
 45. Sullivan, J. J. 1982. Metacyclogenesis of *Trypanosoma cruzi* in vitro: a simplified procedure. *Trans. R. Soc. Trop. Med. Hyg.* **76**:300–303.
 46. Szein, M. B., W. R. Cuna, and F. Kierszenbaum. 1990. *Trypanosoma cruzi* inhibits the expression of CD3, CD4, CD8, and IL-2R by mitogen-activated helper and cytotoxic human lymphocytes. *J. Immunol.* **144**:3558–3562.
 47. Tanowitz, H. B., D. Simon, J. P. Gumprecht, L. M. Weiss, and M. Wittner. 1990. Gastrointestinal manifestations of Chagas' disease, p. 56–75. In V. K. Rustgi (ed.), *Gastrointestinal infections in the tropics*. Karger, Basel.
 48. Tarleton, R. L. 1988. *Trypanosoma cruzi*-induced suppression of IL-1 production. I. Evidence for the presence of IL-2-producing cells. *J. Immunol.* **140**:2763–2768.
 49. Tarleton, R. L. 1988. *Trypanosoma cruzi*-induced suppression of IL-2 production. II. Evidence for a role for suppressor cells. *J. Immunol.* **140**:2769–2773.
 50. Tarleton, R. L., B. H. Koller, A. Latour, and M. Postan. 1992. Susceptibility of β_2 -microglobulin-deficient mice to *Trypanosoma cruzi* infection. *Nature (London)* **356**:338–340.
 51. Tarleton, R. L., and D. W. Scott. 1987. Initial induction of immunity, followed by suppression of responses to parasite antigens during *Trypanosoma cruzi* infection of mice. *Parasite Immunol.* **8**:579–589.
 52. Torrico, F., H. Heremans, M. T. Ribera, E. Van Marck, A. Billiau, and Y. Carlier. 1991. Endogenous IFN-gamma is required for resistance to acute *Trypanosoma cruzi* infection in mice. *J. Immunol.* **146**:3626–3632.
 53. WHO Expert Committee. 1991. Control of Chagas disease. WHO Technical Report Series 811. World Health Organization, Geneva.
 54. Wirth, J. J., F. Kierszenbaum, G. Sonnenfeld, and A. Zlotnik. 1985. Enhancing effects of gamma interferon on phagocytic cell association with and killing of *Trypanosoma cruzi*. *Infect. Immun.* **49**:61.
 55. Yamamura, M., K. Uyemura, R. J. Deans, K. Weinberg, T. H. Rea, B. R. Bloom, and R. L. Modlin. 1991. Defining protective responses to pathogens: cytokine profiles in leprosy lesions. *Science* **254**:277–279.